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Effects of *Rhizobium meliloti nif* and *fix* Mutants on Alfalfa Root Nodule Development

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Ineffective alfalfa nodules were examined at the light and electron microscope level after inoculation with *Rhizobium meliloti* strains with mutations in *nif* and *fix* genes. All the mutant strains induced nodules that contained elongated bacteroids within the host cells, but the bacteroids quickly senesced. The nodules were small and numerous, and the host cells also exhibited symptoms of an ineffective symbiosis. *nifB*, *fixA*, and *fixB* bacteroids appeared to be completely differentiated (by ultrastructural criteria), i.e., as bacteroids developed, they increased in diameter and length and their cytoplasm underwent a change from homogeneous and electron dense to heterogeneous and electron transparent after enlargement. In contrast, *nifA* bacteroids rarely matured to this state. The bacteroids degenerated at an earlier stage of development and did not become electron transparent.

Symbiotic nitrogen fixation is a complex process, involving interactions between the host plant and the endosymbiont that occur during a number of defined steps (34). The molecular genetics of the *Rhizobium*-legume interaction, particularly of the *Rhizobium* partner, has been relatively well characterized at certain points during the development of nitrogen-fixing nodules. These stages in the alfalfa-*Rhizobium meliloti* symbiosis include the elicitation of root hair curling and the initiation of nodule development (17), the determination of nodule host specificity (15, 30), and the regulation and production of nitrogenase polypeptides (29, 35).

Studies on nitrogen fixation in *Rhizobium* and *Bradyrhizobium* species have been facilitated by the identification and characterization of 17 contiguous *nif* genes in *Klebsiella pneumoniae*. Several of the *Klebsiella nif* genes have homologs in *Rhizobium* and *Bradyrhizobium* species including *nifHDK*, the nitrogenase structural genes (24); *nifA*, the positive transcriptional activator for *nifHDK* and other *nif* and *fix* genes (9, 27, 29, 35); *nifB* and *nifE*, genes involved in the synthesis of the nitrogenase iron-molybdenum cofactor FeMoco (3, 10, 11, 20, 23); and *nifF* (13) and *nifJ* (20), genes which code for nitrogenase-specific electron transport proteins.

Despite progress in elucidating the molecular genetics of *nif* gene organization and regulation in *Rhizobium* and *Bradyrhizobium* species, other aspects of the symbiosis are poorly understood at the molecular level. Several developmental features of the symbiosis have been characterized on the basis of mutations which block the differentiation of fully effective root nodules. Strains carrying such mutations are said to have a Fix$^-$ phenotype because they induce the formation of nodules that fail to fix nitrogen. Some of these Fix$^-$ mutants appear to be blocked in early stages of the developmental pathway and have been classed more specifically as nodule development *(ndv)* mutants. These include noninvasive *Rhizobium phaseoli* mutants (33) and noninfecting *R. meliloti*, resulting from mutations in genes that exhibit homology to *chvAB* of *Agrobacterium tumefaciens* (6). The exopolysaccharide-deficient mutants or *exo* mutants of *R. meliloti* (8, 16) that elicit nodule formation without bacterial penetration may also fall into this category. Others have been designated *dif* mutants, i.e., nodule differentiation mutants, but induce a similar phenotype (28). In contrast, many Fix$^-$ mutants appear to be blocked in later stages of development. These mutants induce nodules that contain infection threads and bacteroids, but frequently the bacteroids senesce early and never fix nitrogen. Some nodules may have near wild-type levels of leghemoglobin (1) but still remain ineffective.

Upstream of *nifH* and adjacent to *nifA* on the *R. meliloti* megaplasmid (pSym1) are the *fixABCX* genes which are transcribed as a single operon from *A* to *X* (2, 7, 27). The *fixABCX* transcript, along with the *nifHDK* transcript, are among the most abundant transcripts in *R. meliloti* bacteroids (4). The operon is transcribed under the control of *nifA* (29) and has a canonical *nifA*-regulated promoter (2, 7). The *fixABC* genes do not exhibit homology to *Klebsiella nif* genes at the levels of Southern DNA-DNA hybridization or DNA or protein sequence analysis (7), and the functions of the *fixABC* products are unknown. On the other hand, the *fixX* gene is highly homologous to ferredoxins found in other nitrogen-fixing species such as *Azotobacter vinelandii* (7).

To understand the function of the *fixABCX* operon in *R. meliloti*, we examined the structure of nodules induced by mutants in the *fixA* and *fixB* genes to determine the stage of

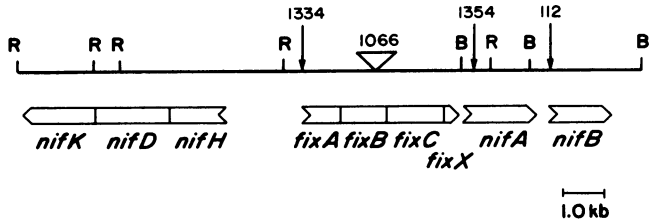

FIG. 1. Map of a region of the *R. meliloti* megaplasmid. The numbers above the arrows refer to the mutant strains analyzed in this study. Symbols: ↓, transposon Tn*5* insertion; ▽, endogenous insertion sequence ISRm1. B, *Bam*HI; R, *Eco*RI; kb, kilobase.

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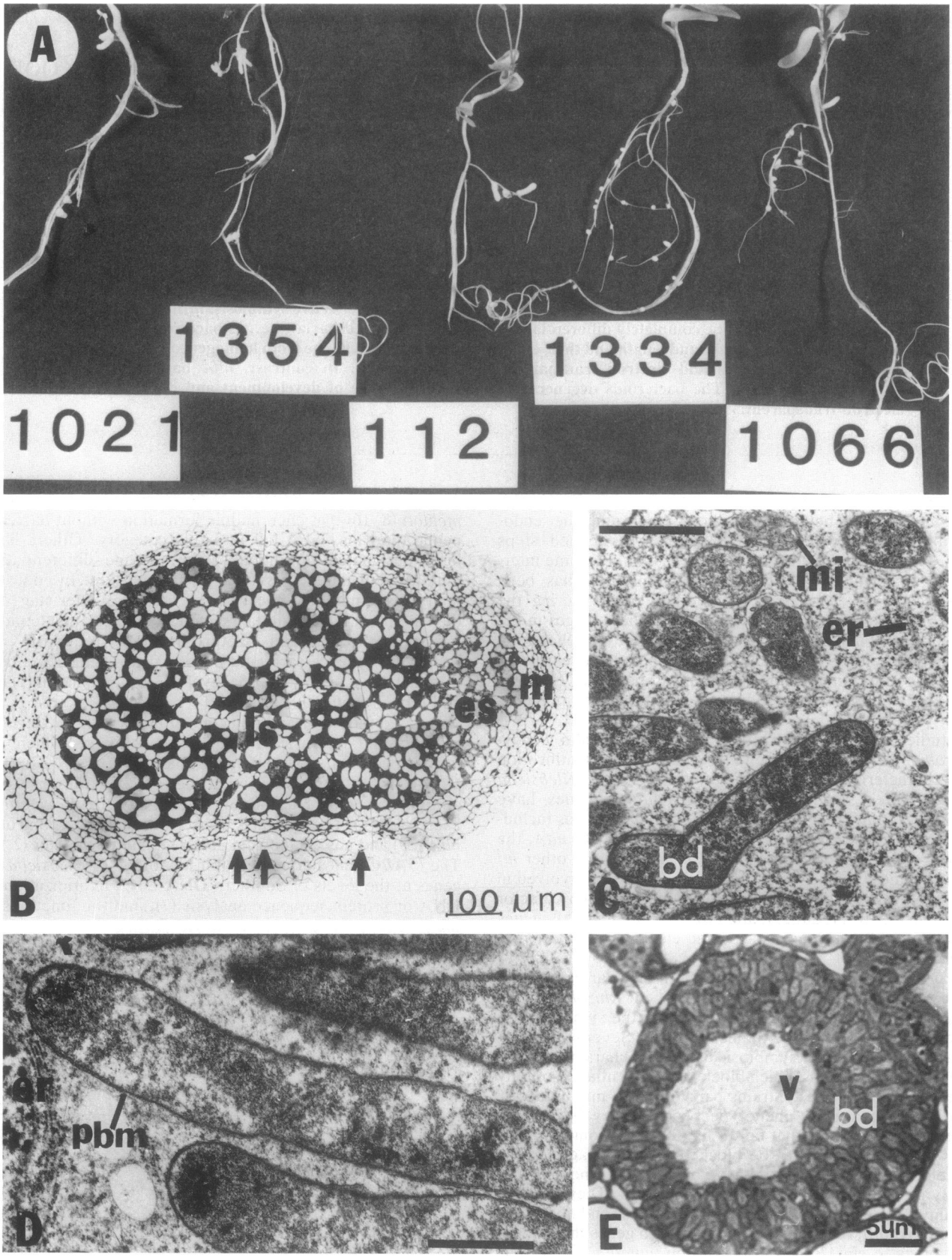
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nodule development arrest. An ultrastructural analysis of this type is useful to illustrate correlations between well-defined genetic lesions and structural abnormalities of nodules. Similar correlations have been made with *R. meliloti* bearing mutations in the *nifHDK* operon (12).

Structural studies of nodules formed in response to *R. meliloti nifA* and *nifB* mutants are included in this study. The nodules elicited by *fixA*, *fixB*, and *nifB* mutants and those induced by *nifA* mutants showed distinct histological and ultrastructural differences from wild type-induced nodules. Moreover, the bacteroids which differentiate from mutant rhizobia resulting from lesions in each of the three operons, *fixABCX*, *nifA*, and *nifB*, exhibited a definite ultrastructural phenotype within alfalfa nodule cells, with *nifA* mutant bacteroids having the most severely affected phenotype.

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* 1021 is a symbiotically effective, streptomycin-resistant derivative of strain SU47 and is the parent strain used to generate the mutants (19). The various *R. meliloti* mutants used in this study (Fig. 1) were constructed in the laboratory of F. M. Ausubel and are described elsewhere (3, 7).

Plant material. Seedlings of alfalfa *Medicago sativa* L. cv. Iroquois were sprouted on slants in test tubes containing Jensen agar and inoculated as described previously (19). The plants were grown under controlled conditions of 16-h, 22°C day and 8-h, 19°C night in a Conviron growth chamber.

Light and electron microscopy. Preparation for light and electron microscopy followed procedures previously described (12). Approximately 20 to 30 alfalfa root nodules were collected and fixed for each mutant strain at 2, 3.5, or 6 weeks after inoculation. No fewer than 10 nodules were examined at the light microscope level, and 5 to 6 of these were analyzed further by electron microscopy.

RESULTS

Mutations which lie upstream of the nitrogenase structural genes (*nifHDK*) were constructed in regions of the *R. meliloti* symbiotic megaplasmid (pSym1) (Fig. 1). We investigated four of these mutant strains in detail. Three of the mutant strains (Rm1354, Rm1334, and Rm112) were constructed by site-directed Tn*5* mutagenesis (25); the fourth (Rm1066) was the result of an insertion of ISRm1, an endogenous insertion sequence element (26).

As shown in Fig. 1, Tn*5* in strain Rm1354 maps to a region homologous to the *K. pneumoniae nifA* gene (29). Tn*5* mutation 112 maps near the 5′ end, most likely in the promoter, of a gene homologous to *K. pneumoniae nifB* (F. M. Ausubel, personal communication). In strain Rm1334, Tn*5* is inserted in *fixA*, and in strain Rm1066, ISRm1 is inserted in *fixB*. All four mutant strains induced ineffective nodules, i.e., had a Nod^+ Fix^- phenotype, on alfalfa roots (Fig. 2A). The structural phenotypes elicited by the four mutant strains were compared with nodules induced by the wild-type (Rm1021) strain. We restricted our analysis to an investigation of bacteroids in the late symbiotic zone because it is within this zone that the mutant-induced nodules demonstrate the most profound differences from those elicited by the wild-type strain.

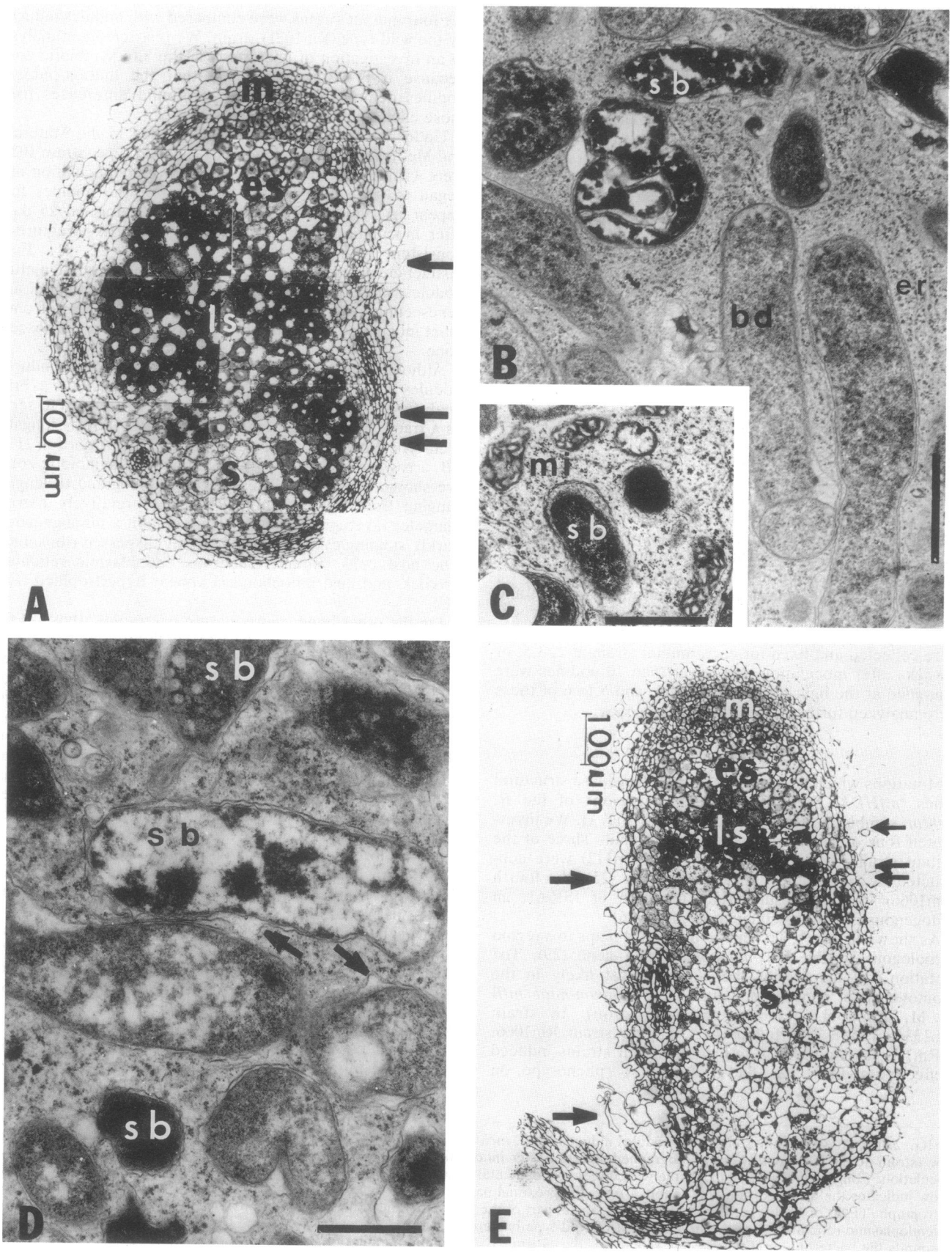
Under the growth conditions described in the Materials and Methods, nodules induced by the wild type, strain 1021, were visible on alfalfa roots 10 days after inoculation and began to fix nitrogen 2 to 4 days after the nodules first appear. A typical effective alfalfa nodule formed 25 days after inoculation is illustrated in Fig. 2B. Its structure is consistent with previous reports (12, 14, 22, 31). Four distinct developmental zones are usually observed in mature nodules: meristematic, early symbiotic, late symbiotic, and senescent zones. The nodule presented in Fig. 2B is from a plant inoculated 3.5 weeks previously and lacks a senescent zone.

Although the details of the structure of wild type-induced nodules have been described before, we include a brief analysis of the ultrastructure of elongate bacteroids to serve as a frame of reference for the studies to follow. Elongate, wild-type Rm1021 bacteroids from the distal portion (Fig. 2B, arrow) of the nitrogen-fixing zone (late symbiotic zone) are shown in Fig. 2C. These rhizobia elongated to lengths ranging from 3 to 8 μm and were of relatively narrow diameter (averaging 0.5 μm). They exhibit a homogeneous, darkly staining cytoplasm with evenly dispersed ribosomes. The host cells contain numerous endoplasmic reticulum profiles, and host mitochondria appear hypertrophied (Fig. 2C).

On the other hand, more mature bacteroids, those in the more proximal region of the late symbiotic zone (Fig. 2B, double arrows), are wider (approximately 1 μm), and their cytoplasms are significantly patchier owing to condensation of electron-dense material within individual bacteroids (Fig. 2D). Individual bacteroids are surrounded by a peribacteroid membrane which in alfalfa nodules is closely appressed to the bacterial outer surface. Mitochondrial cristae are not hypertrophied in this zone, and fewer endoplasmic reticulum profiles are observed. At the light microscope level, bacteroids located in the proximal region of the late symbiotic zone (Fig. 2B, double arrows) of the nodule appear lightly colored after staining with toluidine blue when compared with bacteroids in other developmental zones (Fig. 2E). This staining pattern also demonstrates the more transparent nature of these bacteroids.

The senescent zone of the nodule is the region where both the host cells and the enclosed endosymbiont exhibit progressive deterioration. The first morphological manifestation of senescence is the pulling away of the peribacteroid membrane from the bacteroid. Under transmission electron microscopy (TEM), the bacteroids frequently become very electron dense as they senesce and eventually degenerate such that only empty membranes remain. The host-cell

FIG. 2. (A) Alfalfa root systems with nodules induced by *R. meliloti nifAB* and *fixAB* mutants compared with those elicited by the wild type (strain 1021). The roots were photographed 4 weeks after inoculation. (B to E) Wild type-induced nodules. (B) At 3.5 weeks after inoculation. Longitudinal section of nodule with three developmental zones: m, meristematic; es, early symbiotic; ls, late symbiotic. Single arrow indicates the distal portion and double arrows the proximal part of the late symbiotic zone. Bar, 100 μm. (C) Transmission electron micrograph (TEM) of wild-type bacteroids from the distal part of the late symbiotic zone. Abbreviations: mi, mitochondrion; bd, bacteroid; er, endoplasmic reticulum. Bar, 1 μm. (D) TEM of wild-type bacteroid from the late symbiotic zone. The peribacteroid membrane (pbm) surrounds the bacteroid. er, Endoplasmic reticulum. Bar, 1 μm. (E) Light micrograph of a section through a nodule stained with toluidine blue. Abbreviations: v, vacuole; bd, bacteroid. Bar, 5 μm.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cytoplasm generally senesces soon after bacteroid degeneration. Details regarding alfalfa nodule senescence have been presented previously (21, 32).

Strain 1354 (*nifA*). The gross appearance of Rm1354-induced nodules has been described by Zimmerman et al. (35). Others (5, 9, 18) have noted that nodules induced by *nifA* mutants contain smaller and fewer bacteroids. The nodules were white or straw colored and usually clustered together in groups of four to five along the root (Fig. 2A). The nodules varied in size; some were less than 1 mm in length, while others were as elongate as effective nodules, approximately 2 to 3 mm. Nodules induced by Rm1354 followed a developmental time course identical to that of the wild-type controls, i.e., nodules appeared approximately 10 days after inoculation.

We examined nodules of different ages and found that they exhibited a variable phenotype depending on their size or age. Some small (1 to 2 mm) nodules formed 2 to 3 weeks after inoculation resembled wild-type nodules when viewed in longitudinal section; the degree of development of the meristematic and early symbiotic zones was comparable (Fig. 3A). However, the late symbiotic zone was not as extensive compared with that zone in wild type-induced nodules. Many of the cells interspersed in the late symbiotic zone contained senescent bacteroids, and in addition, a significantly larger senescent zone was observed in nodules induced by *nifA* mutant *R. meliloti*.

In longitudinal sections of older, more elongated nodules, the senescent region became even more apparent (Fig. 3E, large arrows). The meristem continued to function, however, and 8-week-old nodules occasionally reached lengths of 4 to 5 mm, frequently becoming bifurcated.

Host cells of the late symbiotic zone (Fig. 3E, small arrows) in *nifA*::Tn*5*-induced nodules, when examined at the electron microscope level, showed a number of deviations from the condition observed for nodules infected with wild-type *R. meliloti*. Elongated bacteroids were present, but the bacteroids rarely attained the dimensions or appearance of wild-type bacteroids at a comparable stage of development (Fig. 3B to D). In our observations, we found very few *nifA*::Tn*5* bacteroids that were 1 μm in diameter and exhibiting electron-transparent cytoplasm. Most *nifA*::Tn*5* bacteroids were narrow (0.5 μm in diameter), electron dense, and had evenly dispersed, homogeneous, nonpatchy cytoplasm (Fig. 3B, bd). Within the same zone and adjacent to host cells containing homogeneous-appearing bacteroids, nodule cells contained bacteroids which exhibited symptoms of senescence such as the pulling away or rupture of the peribacteroid membrane (Fig. 3D, arrows) and an obvious increase in electron opacity (Fig. 3B and C, sb). The latter symptom was taken as presumptive of bacteroid death and was found frequently in the proximal region of the late symbiotic zone.

Changes in host-cell organelles were also noted. Numerous rough endoplasmic reticulum profiles were evident as was an altered appearance of mitochondrial cristae (Fig. 3B and C). Similar-appearing mitochondria and increased rough endoplasmic reticulum profiles were observed in cells of nodules induced by *nifHDK* mutants (12). Moreover, numerous starch granules lined the periphery of host cells (data not shown).

Strain 112 (*nifB*). Nodules induced by *nifB*::Tn*5* (mutant 112) were variable in size, but frequently were 3 to 6 mm long, longer than any of the other nodules in this study and significantly longer than comparably aged wild type-induced nodules (Fig. 2A). However, some nodules did not elongate beyond 1 mm in length. Like the other ineffective nodules, Rm112-induced nodules were clustered along the roots. Although most nodules were white, a few exhibited a slight pink coloration. Nodules appeared on alfalfa roots 10 to 14 days after inoculation. This is comparable to the time required for wild-type *R. meliloti* strains to induce nodule development.

The histology of elongate *nifB* mutant-induced nodules 16 days after inoculation was similar to that observed in control nodules. Longitudinal sections of mutant-elicited nodules are presented in Fig. 4A. The only difference from wild type-induced nodules observed was an abundance of starch grains in host cells in the proximal region (Fig. 4A, double arrow) of the nodule (Fig. 4B). Mutant *nifB* bacteroids from this zone, when examined at the electron microscope level, were essentially identical in dimension and appearance to wild-type bacteroids (Fig. 4C and D). The cytoplasm of late symbiotic zone bacteroids was electron transparent with some slight heterogeneity, and the bacteroid dimensions were comparable to those of the wild-type strain. Loosening of the peribacteroid membrane was observed in addition to a few senescent bacteroids (Fig. 4C). However, nodules that did not elongate beyond 1 to 2 mm consisted mainly of senescent host cells (data not shown).

We also examined nodules 25 to 42 days after inoculation. These nodules were elongated, but most of the nodule host cells contained senescent bacteroids. An extremely limited zone of bacteroid differentiation (late symbiotic zone) was present in older nodules. Other than containing an increased number of starch grains, the cytoplasmic constituents of host cells also appeared similar to those described for host cells inoculated with wild-type bacteria.

Strain 1334 (*fixA*). Alfalfa nodules induced by mutants in *fixA* (Rm 1334) were small, rarely exceeding 2 mm in length, white or gray-green (presumably from degradation products of leghemoglobin), almost spherical in shape, and numerous. The nodules generally were found scattered along the lateral roots rather than clustered together (Fig. 2A). Mutant-induced nodules appeared on alfalfa roots at the same time (approximately 10 days after inoculation) as did control nodules.

Light micrographic examination of longitudinal sections of nodules indicated that there was extremely truncated development of the meristematic, early symbiotic, and late symbiotic zones (Fig. 5A). All three histological zones appeared compressed, and as a consequence, very few host cells

FIG. 3. Alfalfa nodules induced by strain 1354 (*nifA*::Tn*5*). (A) Longitudinal section of a nodule 3 weeks after inoculation. Single arrow indicates the distal portion and double arrows the proximal part of the late symbiotic zone. All four developmental zones (see Fig. 2B for legend) are present. S, Senescent zone. Bar, 100 μm. (B) TEM of *nifA*::Tn*5* (Rm1354) bacteroids (bd) from the proximal part of the late symbiotic zone of a nodule formed 2 weeks after inoculation. Several bacteroids have senesced (sb). er, Endoplasmic reticulum. Bar, 1 μm. (C) Host-cell mitochondria (mi) exhibit hypertrophied cristae. Bar, 1 μm. (D) TEM of nodule cell containing bacteroids or senescent bacteroids (sb) with discontinuous or loosened peribacteroid membranes (arrows). Bar, 1 μm. (E) Longitudinal section of a nodule formed 6 weeks after inoculation. The late symbiotic (ls) zone is limited, and the distal (single arrow) and proximal (double arrows) portions of the late symbiotic zone are indicated. The senescent zone (s) is delimited by the large arrows to the left of the nodule. Bar, 100 μm.

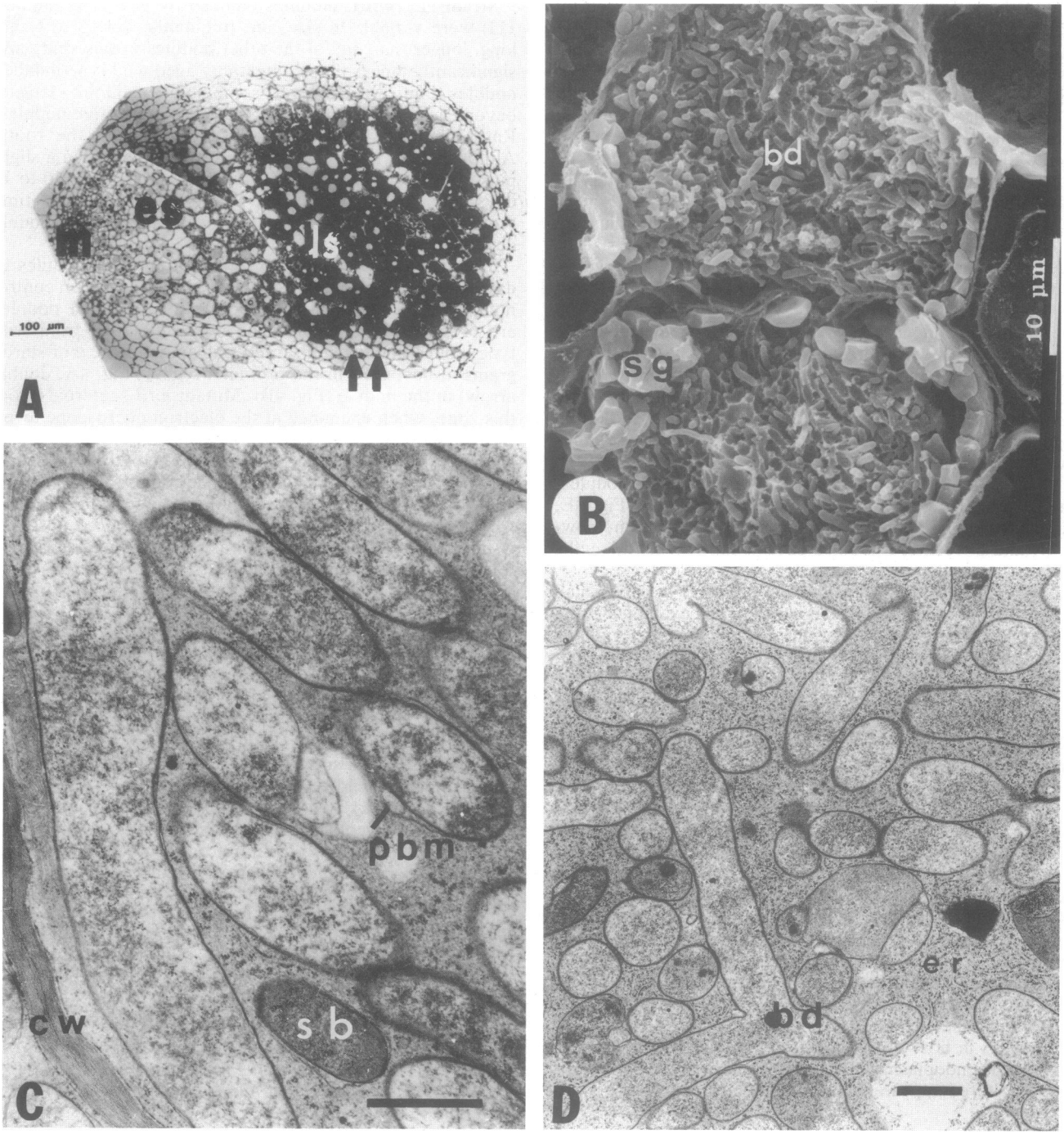


FIG. 4. Alfalfa nodules induced by strain 112 (*nifB*::Tn*5*). (A) Longitudinal section of a nodule formed 16 days after inoculation. Three developmental zones are present (m, mitochondria; es, early symbiotic zone; ls, late symbiotic zone). Bar, 100 μm. (B) Scanning electron micrograph of late symbiotic zone nodule cells containing elongate bacteroids (bd) and numerous starch grains (sg) which line the periphery. Bar, 10 μm. (C) TEM of bacteroids from the distal portion of the late symbiotic zone. Many show loosening of the peribacteroid membrane (pbm). Abbreviations: sb, senescent bacteroids; cw, cell wall. (D) TEM of nodule cell containing bacteroids (bd) and numerous profiles of endoplasmic reticulum (er). Bar, 1 μm.

contained elongated bacteroids. The transition from the early symbiotic zone to the senescent region was abrupt, occurring within one to two cells. Most of the nodules consisted of host cells that were senescent and that contained degenerated bacteroids.

Like mutant *nifA* bacteroids, bacteroids in *fixA*::Tn*5*-induced nodules elongated but, in contrast, were more likely to exhibit a wild-type, Rm 1021-like cytoplasm (Fig. 5B) in the distal region of the late symbiotic zone (Fig. 5A, arrow). The bacteroids were electron transparent and of similar diameter as wild-type bacteroids. Bacteroids in the proximal region of the same zone (Fig. 5A, double arrows) exhibited such symptoms of senescence as an increased electron density and loosening of the peribacteroid membrane (Fig. 5C, arrows). Numerous rough endoplasmic reticulum and Golgi body profiles characterized the host-cell cytoplasm, and mitochondrial cristae were hypertrophied (Fig. 5C).

Strain 1066 (*fixB*). Nodules elicited by *fixB*::Tn*5* (Rm1066) were small (1 to 3 mm), straw colored, and clustered on lateral roots (Fig. 2A). These nodules were frequently darkly colored at the proximal end and occasionally were as long as 4 mm. The time period for nodule development followed kinetics similar to those described earlier for the other mutants.

Like *fixA*::Tn*5*-induced nodules, nodules formed in response to strain 1066 (*fixB* mutants) have limited development of an elongate bacteroid zone (late symbiotic zone) 2 to 3 weeks after inoculation (Fig. 5F). Most of the bacteroid-containing cells are at the distal end of the nodule, again indicating a curtailed development of the late symbiotic zone.

Breakage and pulling away of the peribacteroid membrane and degeneration of bacteroids was observed under TEM (Fig. 5D and E). Many *fixB* bacteroids accumulated electron-dense deposits (presumably polyphosphate) that were usually localized to one end (data not shown). In some nodules, the mature late symbiotic zone bacteroids (Fig. 5F, arrow) developed an electron-transparent cytoplasm similar to that of Rm1021 bacteroids (Fig. 5D). However, host cells are one to two cells removed from this zone (Fig. 5F, double arrows) already exhibited signs of bacteroid degeneration (Fig. 5E).

The host-cell cytoplasm appeared morphologically intact even in regions where the bacteroids had begun to degenerate (Fig. 5E). We observed that there was considerable proliferation of host membranes within cells. As was found for the other ineffective nodules, numerous amyloplasts lined the periphery of the host cells.

DISCUSSION

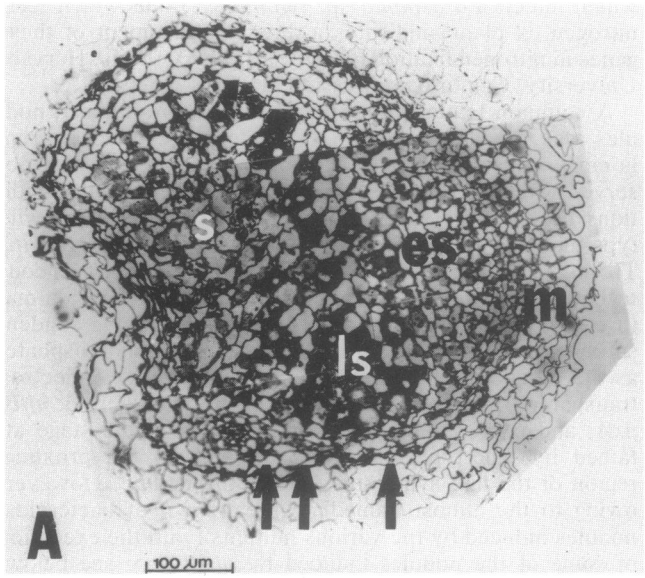
The goal of this study was to investigate the structural phenotype of alfalfa root nodules elicited by *R. meliloti* strains carrying single mutations in the *nif*-specific regulatory gene, *nifA*, in the *nifB* gene (whose homolog in *K. pneumoniae* participates in FeMoCo synthesis), and in symbiotic genes of unknown function, *fixA* and *fixB*. It was hoped that a study of this nature might suggest a possible function of the *fixABCX* operon in nitrogen fixation. Although the *fixABC* genes are essential for an effective symbiosis, they are not homologous to any *K. pneumoniae nif* genes at the DNA or protein sequence level and do not complement *K. pneumoniae nif* mutations (7). The *fixX* gene codes for a ferredoxinlike protein (7); however, there is no direct biochemical evidence which shows that this ferredoxin is involved in electron transport to nitrogenase. The Fix$^-$ phenotype of *fixBC* mutants in a *Bradyrhizobium* sp. which infects *Parasponia* sp. *(Ulmaceae)* and which fixes nitrogen ex planta indicates a direct involvement of these genes in nitrogen fixation (C. D. Earl, Ph.D. thesis, Harvard University, Cambridge, Mass., 1986).

A common feature found in all the mutant-induced nodules was the differentiation of bacteroid cytoplasm from homogeneous to heterogeneous. However, this was observed rarely for *nifA* bacteroids under the growth conditions used in this study. As reported previously for wild type-induced nodules and those elicited by mutants carrying Tn*5* in the *nifHDK* operon (12), a change from homogeneous to heterogeneous cytoplasm is observed as bacteroids mature. The increased heterogeneity may be related to condensation of nuclear or storage material (such as polyphosphate) as the remainder of the cytoplasm becomes more electron transparent owing to an enlargement of the bacteroid. *nifB*, *fixA*, and *fixB* mutant bacteroids develop to the stage attained by wild-type Rm1021 bacteroids in the proximal region of the late symbiotic zone of the nodule. However, owing to the almost immediate breakdown of bacteroids, nodules induced by the various mutants (with the exception of some of the nodules induced by *nifB*::Tn*5*; see below) exhibited limited development of the distinctive zones which characterize alfalfa root nodules.

The effects on nodule development of the one *R. meliloti nifB* mutant we examined were less deleterious than those generated by the *nifA*, *fixA*, and *fixB* mutants. We found that *nifB*::Tn*5*-induced nodules were structurally very similar to wild type-elicited nodules, especially in the early stages of development. *nifB* mutant bacteroids differentiate to the mature bacteroid state and may even persist for an extended period of time before degeneration. This particular *nifB* mutation (Rm112) lies near the 5′ end of the gene that is approximately 50% homologous with the *K. pneumoniae nifB* gene and 67% homologous with the *Rhizobium leguminosarum fixZ* gene (3). Other *nifB* mutants that result from transposons positioned in the middle of the gene may elicit a more deleterious effect on nodule development.

Single mutations in either the *fixA* or *fixB* gene also result in a Nod$^+$ Fix$^-$ phenotype. Biochemical analyses by Zimmerman et al. (35) showed that mutants in the *fixABCX* operon accumulate *nifHDK* mRNA and polypeptides. In addition, approximately 64% of wild-type leghemoglobin levels (as measured by the apoprotein) are found in nodules induced by strains Rm1066 and Rm1334 (35). Thus, much of the biochemical machinery up to the point of initiating nitrogen fixation appears to be intact in *fixA* and *fixB* bacteroids. Indeed, our investigation showed that the bacteroids attain an ultrastructural appearance, albeit transient, comparable to that of wild-type bacteroids. This morphological appearance may be coupled to the biochemical state of the bacteroid just before the onset of nitrogen fixation. However, *fixB* and *fixA* mutant bacteroids quickly lose morphological integrity and senesce. The host cells also senesce rapidly, and hence the nodules are very small and the late symbiotic zone forms a limited portion of the mature nodule.

These ultrastructural studies indicate that there are similarities among the nodule phenotypes elicited by the *nif* mutants studied previously (12) and by *R. meliloti fixA* and *fixB* mutants. The observations that the *fixABC* genes are conserved among *Rhizobium* species and that *fixBC* mutants of "*Bradyrhizobium parasponiae*" Rp501 are Fix$^-$ even when under depressing conditions in vitro also suggest that the *fixABC* products are involved directly in the nitrogen fixation process. Because *fixX* has been shown to code for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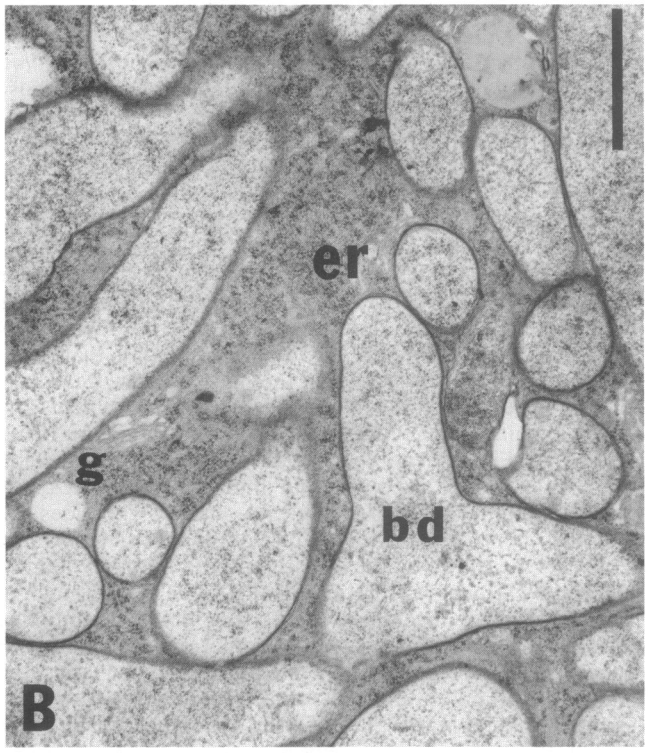
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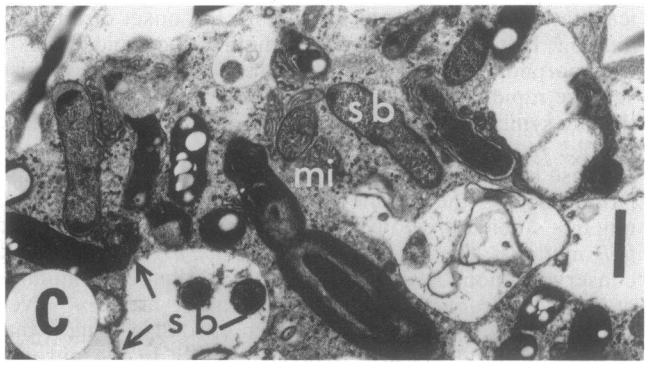
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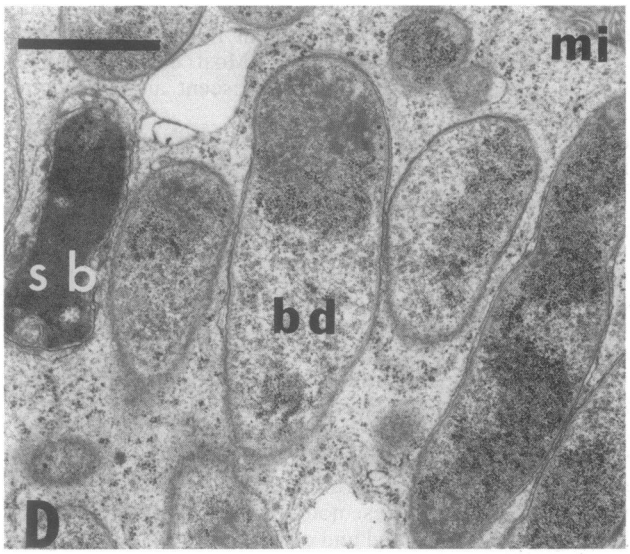
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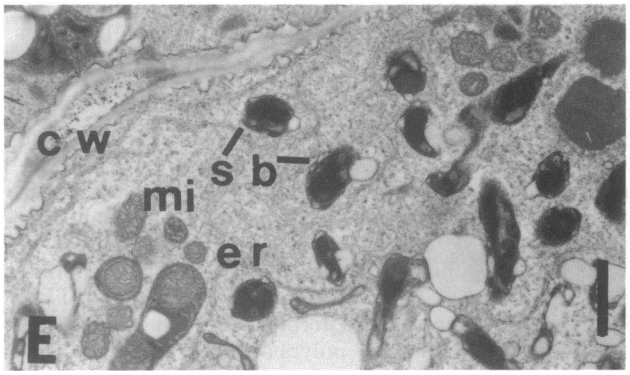
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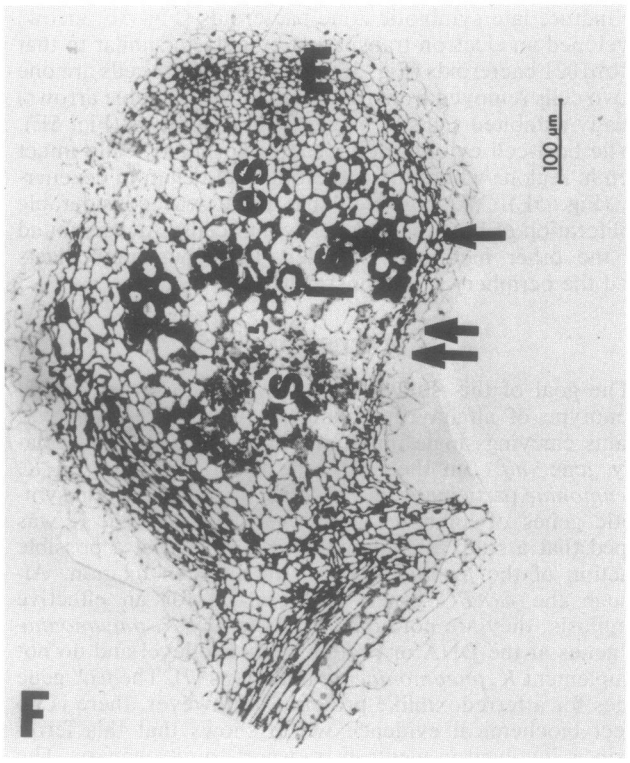
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FIG. 5. (A to C) Alfalfa nodules induced by strain 1334 (*fixA*::Tn*5*). (A) Longitudinal section of a nodule formed 4 weeks after inoculation. Most of the nodule is senescent (s). Single arrow indicates the distal portion and double arrows the proximal part of the late symbiotic (ls) zone. es, Early symbiotic zone; m, mitochondria. Bar, 100 μm. (B) TEM of bacteroids (bd) in the distal part of the late symbiotic zone. Golgi bodies (g) and numerous endoplasmic reticulum profiles (er) are present in the host cell. Bar, 1 μm. (C) TEM of host cell in proximal part of the late symbiotic zone. Bar, 1 μm. The bacteroids are senescent (sb), and the host mitochondria (mi) are hypertrophied. (D to F) Alfalfa nodules induced by *fixB* mutant strain 1066. (D) TEM of bacteroids (bd) in the distal region of the late symbiotic zone. Some are senescent (sb). Bar, 1 μm. (E) TEM of host cell in proximal part of the late symbiotic zone. The host cell contains senescent bacteroids (sb), mitochondria (mi), and endoplasmic reticulum (er) and is bounded by a cell wall (cw). Bar, 1 μm. (F) Longitudinal section of a nodule formed 3.5 weeks after inoculation with strain 1066. Most of the nodule is senescent (s), but the other developmental zones are present (m, es, ls). Single arrow indicates the distal part and double arrows the proximal part of the late symbiotic zone. Bar, 100 μm.

ferredoxin (7), the *fixABC* gene products also may be involved in electron transport, analogous to the *nifJ* product in *K. pneumoniae*. Moreover, because the insertion elements in strains Rm1334 and Rm1066 could be polar on *fixC* and *fixX*, the observed phenotype of the *fixA* and *fixB* mutants therefore may be due to the loss of electron transport in these mutant bacteroids causing a collapse of the symbiosis. Another possible function for the *fixABC* operon is that the gene products may trigger the next step of bacteroid development and, as a consequence, persistent nodule function.

In contrast, *nifA* mutant (Rm1354) bacteroids synthesize neither mRNA nor polypeptides of the nitrogenase structural genes (the *nifHDK* operon), the *fixABC* operon, and the *nifB* gene (29, 35). Interestingly, the bacteroids also did not mature to a state, by ultrastructural criteria, which is judged as fully differentiated. This suggests that the *nifA* gene product is required for the final stages of bacteroid differentiation as well as induction of other *nif* and *fix* genes. However, the consequences of *nifA*::Tn*5* bacteroid deterioration on nodule development itself are not as severe as the *fixA* and *fixB* mutants. Indeed, many mutant *nifA*-induced nodules are as elongated as wild type-induced nodules (Fig. 2A).

The interruption of *nifA* mutant bacteroid development is not due to nitrogen starvation because signs of bacteroid degradation are apparent as early as 16 days after inoculation in *nifA* mutant-induced nodules and not until 3 to 5 days later in nodules elicited by other *fix* and *nif* mutants. Such a severe effect on bacteroid phenotype resulting from a *nifA* mutation suggests that there are as of yet undiscovered symbiotic genes which are also regulated by *nifA*. These may be genes involved in bacteroid differentiation and persistence. It may be possible to predict the function of these genes by using the type of correlation described here between known genetic lesions and ultrastructure.

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